SPECIFICATION

MODIFIED THERMOSTABLE DNA POLYMERASE

TECHNICAL FIELD

The present invention relates to a thermostable DNA polymerase with enhanced amplification efficiency and/or improved fidelity in polymerase chain reaction (PCR), and to a process for production thereof. The present invention further relates to a method for amplifying nucleic acid using the thermostable DNA polymerase, and a reagent kit comprising the thermostable DNA polymerase.

In resent years, PCR has been one of the essential techniques for research and testing in the fields of biochemistry, molecular biology and clinicopathology. A feature of PCR is that the reaction is carried out using a thermostable DNA polymerase. The DNA polymerases most frequently utilized currently are, mainly, thermostable DNA polymerases called "Pol I-like", such as a thermostable DNA polymerase derived from Thermus aquaticus (Taq DNA polymerase) and a thermostable DNA polymerase derived from Thermus thermophilus (Tth DNA polymerase). The advantageous characteristics of Pol I-like DNA polymerases are high amplification efficiency and easiness to set conditions. However, these enzymes have a

defect of low fidelity in nucleic acid incorporation during amplification and are considered to be unsuitable for use in the case of cloning the amplified DNA.

Other known polymerases are "α-like" DNA polymerases derived from hyperthermophilic archaea, such as a thermostable DNA polymerase derived from Pyrococcus furiosus (Pfu DNA polymerase, WO92/09689, Japanese Unexamined Patent Publication No. 1993-328969), a thermostable DNA polymerase derived from Thermococcus litoralis (Ti(Vent) polymerase, Japanese Unexamined Patent Publication No. 1994-7160), and a thermostable DNA polymerase derived from Pyrococcus kodakaraensis KOD1 (former name: Pyrococcus sp. KOD1)(KOD DNA polymerase, Japanese Unexamined Patent Publication No. 1995-298879). Advantageous characteristics of α-like DNA polymerases are that the polymerases have 3′-5′ exonuclease activity (proof-reading activity) and high fidelity in nucleic acid incorporation as compared with Pol I-like DNA polymerases such as Taq DNA polymerase.

However, α-like DNA polymerases have problems such as insufficient PCR amplification efficiency. Furthermore, most of the α-like DNA polymerases have the disadvantage that optimal conditions for PCR such as reaction time, enzyme amount and primer concentration are limited to narrow ranges.

The 3′-5′ exonuclease activity level is presumably a cause of the aforementioned problems with PCR amplification using α-like DNA polymerase. Stated more specifically, it is considered that nucleotides are removed from primers etc. by 3′-5′ exonuclease activity during PCR, whereby PCR amplification efficiency is reduced. Further, since α-like DNA polymerase has a 3′-5′ exonuclease activity domain and a DNA polymerase activity domain in a single protein, it is presumed that the interaction between the two activities and the difference between these sites in affinity to nucleic acid also affect PCR amplification.

It is known that highly conserved amino acid regions (EXO I (FIG. 1), EXO II and EXO III) of α-like DNA polymerase might play a significant role in expressing 3′-5′ exonuclease activity (*Gene*, 100, 27-38 (1991), *Gene*, 112, 139-144 (1992)). It is known that XDXEX sequence (D: aspartic acid, E: glutamic acid, each of X: any amino acid) exists in the EXO I region, and aspartic acid (D) and glutamic acid (E) are essential for exhibiting exonuclease activity (Kong, *et al.* (1993), *Journal of Biological Chemistry*, vol. 268, 1965-1975). According to the above publication, exonuclease activity can be reduced to 1/10000 or less by replacement of aspartic acid and glutamic acid in the EXO I region by alanine which is a

neutral amino acid. However, there exists a problem that when exonuclease activity is reduced to 1/10000 or less, high fidelity of DNA replication, which is an advantageous characteristic of α-like DNA polymerases, is also lost simultaneously.

Further, there is a report on an attempt to gradually reduce 3′-5′ exonuclease activity by replacing an amino acid represented by X_1 in the above $X_0DX_1EX_2$ sequence of KOD DNA polymerase with a different amino acid (Japanese Unexamined Patent Publication No. 1998-42871). According to the method disclosed therein, as 3′-5′ exonuclease activity decreases, enhanced PCR efficiency and reduced amplification fidelity are observed simultaneously. Therefore, in the case of replacing the above-mentioned amino acid (X_1), it is important to prepare a modified enzyme whose 3′-5′ exonuclease activity has been reduced within the range that fidelity of enzyme amplification is not impaired. However, enzymes produced according to the above method, such as variants IQ and IK prepared by replacing isoleucine(I) at the 142-position from the 5′-terminal of KOD DNA polymerase by glutamine(Q) and lysine(K) respectively, do not always show high amplification efficiency from low copy number of template DNA and therefore, can not be regarded as variants achieving high PCR efficiency (FIG. 3).

According to Japanese Unexamined Patent Publication No. 1998-42871, it is difficult to produce mutants with enhanced 3′-5′ exonuclease activity (proof-reading activity) by the method disclosed therein.

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The present inventors produced various mutants of KOD DNA polymerase and carried out extensive research to solve the above problems. The present inventors found that when the histidine residue that is the fourth amino acid from glutamic acid(E) of $X_0DX_1EX_2$ sequence in the EXO I region (i.e., at the 147-position; hereinafter sometimes referred to as "H") is replaced by various amino acids, there can be produced thermostable DNA polymerases with different levels of 3′-5′ exonuclease activity, PCR efficiency and fidelity. Based on this finding, the present invention has been accomplished (the motif including this histidine is hereinafter referred to as $DX_1EX_2X_3X_4H$ sequence).

The present invention includes the following subject matters:

1. A modified thermostable DNA polymerase wherein in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino acid) consisting of DX_1E sequence within the EXO I region and a four amino acid length peptide adjacent to said glutamic acid(E) of

thermostable DNA polymerase having 3'-5' exonuclease activity, histidine(H) has been replaced by another amino acid.

2. The modified thermostable DNA polymerase according to item 1, wherein in the $DX_1EX_2X_3X_4H$ sequence, histidine(H) has been replaced by an amino acid selected from the group consisting of aspartic acid, glutamic acid, tyrosine, alanine, lysine and arginine.

3. The modified thermostable DNA polymerase according to item 1 having the following physicochemical properties:

(1) DNA extension rate: at least 20 bases/second; and

(2) thermostability: it is capable of retaining 10% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours.

4. The modified thermostable DNA polymerase according to item 3 having the following physicochemical properties:

(1) DNA extension rate: at least 30 bases/second;

(2) thermostability: it is capable of retaining 40% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours; and

(3) amino acid sequence: in the DIETLYH sequence (D: aspartic acid, I: isoleucine, E: glutamic acid, T: threonine, L: leucine, Y: tyrosine, H: histidine) located

at the 141- to 147-positions in the amino acid sequence of SEQ ID NO: 2, histidine(H) has been replaced by another amino acid.

5. The modified thermostable DNA polymerase according to item 4 having the following physicochemical properties:

(1) DNA extension rate: at least 30 bases/second;

(2) thermostability: it is capable of retaining 60% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours; and

(3) amino acid sequence: in the DIETLYH sequence (D: aspartic acid, I: isoleucine, E: glutamic acid, T: threonine, L: leucine, Y: tyrosine, H: histidine) located at the 141- to 147-positions in the amino acid sequence of SEQ ID NO: 2, histidine(H) has been replaced by another amino acid.

6. The modified thermostable DNA polymerase according to item 5, wherein in the amino acid sequence of SEQ ID NO: 2, histidine(H) at the 147-position has been replaced by an amino acid selected from the group consisting of aspartic acid, glutamic acid, tyrosine, alanine, lysine and arginine.

7. The modified thermostable DNA polymerase according to item 6, wherein in the amino acid sequence of SEQ ID NO: 2, histidine(H) at the 147-position has been replaced by

aspartic acid.

8. The modified thermostable DNA polymerase according to item 6, wherein in the amino acid sequence of SEQ ID NO: 2, histidine(H) at the 147-position has been replaced by glutamic acid.

9. The modified thermostable DNA polymerase according to item 6, wherein in the amino acid sequence of SEQ ID NO: 2, histidine(H) at the 147-position has been replaced by tyrosine.

10. The modified thermostable DNA polymerase according to item 6, wherein in the amino acid sequence of SEQ ID NO: 2, histidine(H) at the 147-position has been replaced by alanine.

11. The modified thermostable DNA polymerase according to item 6, wherein in the amino acid sequence of SEQ ID NO: 2, histidine(H) at the 147-position has been replaced by lysine.

12. The modified thermostable DNA polymerase according to item 6, wherein in the amino acid sequence of SEQ ID NO: 2, histidine(H) at the 147-position has been replaced by arginine.

13. A gene encoding a modified thermostable DNA polymerase wherein in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino acid) consisting of DX_1E sequence within the EXO I

region and four amino acid length peptide adjacent to said glutamic acid(E) of thermostable DNA polymerase having 3'-5' exonuclease activity, histidine(H) has been replaced by another amino acid.

14. The gene according to item 13 which encodes a modified thermostable DNA polymerase having the following physicochemical properties:

(1) DNA extension rate: at least 20 bases/second; and

(2) thermostability: it is capable of retaining 10% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours.

15. The gene according to item 13 which encodes a modified thermostable DNA polymerase having the following physicochemical properties:

(1) DNA extension rate: at least 30 bases/second;

(2) thermostability: it is capable of retaining 40% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours; and

(3) amino acid sequence: in the DIETLYH sequence (D: aspartic acid, I: isoleucine, E: glutamic acid, T: threonine, L: leucine, Y: tyrosine, H: histidine) located at the 141- to 147-positions in the amino acid sequence of SEQ ID NO: 2, histidine(H) has been replaced by another

amino acid.

16. The gene according to item 13 which encodes a modified thermostable DNA polymerase having the following physicochemical properties:

(1) DNA extension rate: at least 30 bases/second;

(2) thermostability: it is capable of retaining 60% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours; and

(3) amino acid sequence: in the DIETLYH sequence (D: aspartic acid, I: isoleucine, E: glutamic acid, T: threonine, L: leucine, Y: tyrosine, H: histidine) located at the 141- to 147-positions in the amino acid sequence of SEQ ID NO: 2, histidine(H) has been replaced by another amino acid.

17. A recombinant DNA vector obtained by inserting the gene of any one of items 13 to 16 into an expression vector.

18. The recombinant DNA vector according to item 17, wherein the expression vector is pLED-MI, pBluescript or their derivatives.

19. A transformant produced by transforming a host cell with the recombinant DNA vector of item 17 or 18.

20. The transformant according to item 19 wherein the host cell is Escherichia coli.

21. A process for producing a modified thermostable DNA polymerase, which comprises culturing the transformant of item 20 and recovering the thermostable DNA polymerase from the culture broth.

22. A method for amplifying or extending nucleic acid, which comprises reacting DNA as a template, one or more kinds of primers, dNTP and the thermostable DNA polymerase of any one of items 1 to 12, thus extending the primer(s) to synthesize DNA primer extension product(s).

23. The method for amplifying nucleic acid according to item 22, wherein the primers are 2 kinds of oligonucleotides, each of the primers being complementary to a DNA extension product of the other primer.

24. The method for amplifying nucleic acid according to item 22, which comprises heating and cooling repeatedly.

25. A reagent kit for amplifying nucleic acid, which comprises 2 kinds of primers, each of the primers being complementary to a DNA extension product of the other primer; dNTP; the thermostable DNA polymerase of any one of items 1-12; divalent ion(s); monovalent ion(s); and a buffer solution.

26. A reagent kit for amplifying nucleic acid, which comprises 2 kinds of primers, each of the primers being complementary to a DNA extension product of the other primer; dNTP; the thermostable DNA polymerase of any one

of items 1-12; magnesium ion; at least one of monovalent ions selected from the group consisting of ammonium ion and potassium ion; BSA (bovine serum albumin); a nonionic surfactant and a buffer solution.

27. A reagent kit for amplifying nucleic acid, which comprises 2 kinds of primers, each of the primers being complementary to a DNA extension product of the other primer; dNTP; the thermostable DNA polymerase of any one of items 1-12; magnesium ion; at least one of monovalent ions selected from the group consisting of ammonium ion and potassium ion; BSA (bovine serum albumin); a nonionic surfactant; a buffer solution and an antibody capable of suppressing at least one activity selected from polymerase activity and 3'-5' exonuclease activity of the thermostable DNA polymerase.

28. A DNA polymerase composition which comprises one or more kinds of modified thermostable DNA polymerases defined in any of items 1-12.

29. A method of producing a mutated DNA which comprises reacting DNA as a template, mutagenesis primers, dNTP and the thermostable DNA polymerase of any one of items 1 to 12, thus extending the primers to synthesize DNA primer extension products.

30. A reagent kit for producing a mutated DNA which comprises mutagenesis primers, dNTP and the thermostable

DNA polymerase of any one of items 1 to 12.

DNA polymerase activity

In the present invention, "DNA polymerase activity" refers to a catalytic activity to template-dependently introduce deoxyribonucleoside-5'-monophosphate into deoxyribonucleic acid by covalently binding α-phosphate of deoxyribonucleoside-5'-triphosphate to the 3'-hydroxyl group of an oligonucleotide or polynucleotide annealed to a template DNA.

If the enzyme activity in a sample is high, activity measurement shall be carried out after the sample is diluted with a storage buffer (for example, 50 mM Tris-HCl(pH8.0), 50 mM KCl, 1 mM DTT, 0.1 % Tween 20, 0.1 % Nonidet P40, 50 % glycerin) . In the present invention, 25 µl of Solution A shown below, 5 µl each of Solutions B and C shown below, 10 µl of sterilized water and 5 µl of an enzyme solution are pipetted into a microtube and reacted at 75°C for 10 minutes. Thereafter, the sample is cooled on ice, and 50 µl of Solution E and 100 µl of Solution D shown below are added and stirred, followed by cooling with ice for 10 minutes. The solution is filtered through a glass filter (Wattman GF/C Filter), and the filter is washed intensively with Solution D and ethanol, and the radioactivity of the filter is counted in a liquid

scintillation counter (Packard) to determine the incorporation of the nucleotide into the template DNA. 1 unit of enzyme activity is defined as the amount of the enzyme that catalyzes an incorporation of 10 nmole of total nucleotides into the acid-insoluble fraction (i.e., DNA fraction which becomes insoluble when Solution D is added) per 30 minutes under the above conditions.

Solution A: 40 mM Tris-HCl buffer (pH 7.5)
16 mM magnesium chloride
15 mM dithiothreitol
100 µg/ml BSA

Solution B: 2 µg/µl activated calf thymus DNA

Solution C: 1.5 mM dNTP (250 cpm/pmol [^{3}H]dTTP)

Solution D: 20% trichloroacetic acid (2 mM sodium pyrophosphate)

Solution E: 1 mg/ml salmon sperm DNA

3′-5′ exonuclease activity

In the present invention, "3′-5′ exonuclease activity" refers to the activity of deleting a 3′-terminal region of DNA to release 5′-mononucleotide. The activity measurement method is as follows: 50 µl reaction solution (120 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 6 mM ammonium sulfate, 1 mM $MgCl_2$, 0.1% Triton X-100, 0.001%

BSA and 5 μg of E. coli DNA labeled with tritium) are pipetted into a 1.5 ml microtube, followed by addition of DNA polymerase. The mixture is reacted at 75°C for 10 minutes and then cooled with ice to terminate the reaction. After 50 μl of 0.1% BSA is added as a carrier to the reaction mixture, 100 μl of a solution containing 10% trichloroacetic acid and 2% sodium pyrophosphate is added and mixed. The mixture is left on ice for 15 minutes and then centrifuged at 12,000 r.p.m. (rotations per minute) for 10 minutes to separate a supernatant from the precipitate. 100 μl of the supernatant is measured for radioactivity in a liquid scintillation counter (Packard) to determine the amount of the nucleotide delivered to the acid soluble fraction.

<u>DNA extension rate</u>

In the present invention, "DNA extension rate" refers to the number of extended nucleotides per one second (bases/second) on an elongation reaction of DNA polymerase. The measurement method is as follows: A reaction solution of DNA polymerase (20 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride, 7.5 mM dithiothreitol, 100 μg/ml BSA, 0.1 mM dNTP, 0.2 μCi [α-^{32}P]dCTP) is reacted at 75°C with single-stranded M13mp18 DNA to which a primer had been annealed. The reaction is terminated by adding

an equal volume (equal to the reaction mixture) of a reaction terminating solution (50 mM sodium hydroxide, 10 mM EDTA, 5% Ficoll, 0.05% Bromophenol Blue). The DNA fragments are sized by electrophoresis on an alkaline agarose gel, and the gel is dried and subjected to autoradiography. As the DNA size marker, labeled λ/HindIII is used. DNA extension rate is determined by measuring the extended DNA size using a band of this marker as an indicator.

Thermostability (residual polymerase activity after heat treatment)

In the present invention, "thermostability" means residual activity after mixing 5 units of DNA polymerase with 100 µl of a buffer (20 mM Tris-HCl (pH 8.8, the pH value determined at 25°C), 10 mM potassium chloride, 10 mM ammonium sulfate, 2mM magnesium sulfate, 0.1% Triton X-100, 0.1 mg/ml BSA and 5 mM 2-mercaptoethanol) and heating the mixture at 95°C for 6 hours. More specifically, thermostability is determined by measuring DNA polymerase activity after the heat treatment and comparing the value with that before the heat treatment.

Fidelity of DNA polymerase

In the present invention, "fidelity of DNA

polymerase" refers to the accuracy in nucleotide incorporation during DNA replication. Ribosomal protein S12 (rpsL) gene derived from E. coli and relating to streptomycin resistance is used as an indicator to evaluate fidelity of DNA polymerase in the present invention. Streptomycin is an antibiotic which inhibits protein synthesis in prokaryote. Streptomycin binds to 30S ribosomal RNA (rRNA) in prokaryote to thereby inhibit the reaction of producing a protein synthesis initiation complex and cause the misreading of genetic code. Streptomycin-resistant strains have a mutation at ribosome protein S12 locus. It is known that this mutation produces pleiotropic effects for enhancing translation fidelity of ribosome, for example, inhibiting suppressor tRNA from reading the end codon. Thus, when PCR amplification is carried out using rpsL gene as a template, a mutation is introduced with a certain probability. When the mutation occurs at amino acid level, the rpsL protein structure will change so that streptomycin may fail to bind to 30S ribosomal RNA (rRNA). Therefore, when the strain is transformed by an amplified plasmid DNA, appearance frequency of streptomycin-resistant strains increases as more mutation is introduced.

Plasmid pMol 21 (described in *Journal of Molecular Biology* (1999) 289, 835-850) is a plasmid

containing rpsL gene and ampicillin resistant gene. The fidelity of DNA replication can be determined by a method comprising the following steps:

(1) designing a primer set (one of the primers is biotinylated and the restriction site of MluI is introduced to the primers) for PCR amplification on the ampicillin resistant gene of the plasmid pMol 21;

(2) amplifying the full-length plasmid by PCR using a thermostable DNA polymerase;

(3) purifying the amplified plasmid using streptavidin beads;

(4) cutting out the amplified plasmid from the streptavidin beads using restriction enzyme MluI;

(5) ligating the ends to form a circular plasmid using DNA ligase to transform E. coli;

(6) innoculating the transformant E. coli into two kinds of plates (one containing ampicillin and the other containing ampicillin and streptomycin); and

(7) calculating the ratio of numbers of colonies appearing on the plates.

The modified thermostable DNA polymerase of the present invention is an enzyme wherein in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, each of X_1, X_2, X_3 and X_4: any amino acid) consisting of DX_1E

sequence within the EXO I region and four amino acid length peptide adjacent to said glutamic acid(E) of thermostable DNA polymerase having 3'-5' exonuclease activity, histidine(H) has been replaced by another amino acid. The definition of the EXO I region slightly varies in different reports. However, the DX_1E sequence is commonly included in the EXO I region and the C-terminal of the EXO I region is any one of X_2, X_3 and X_4 in a various reports.

There is no restriction on the origin of thermostable DNA polymerase having the $DX_1EX_2X_3X_4H$ sequence consisting of a part of the C-terminal region of the EXO I region and an amino acid sequence adjacent thereto. Specific examples of thermostable DNA polymerase are KOD DNA polymerase derived from Pyrococcus kodakaraensis KOD1, thermostable DNA polymerase derived from Pyrococcus furiosus, and thermostable DNA polymerase derived from Thermococcus litoralis. According to some recent classification schemes, Pyrococcus kodakaraensis is classified as a member of Thermococcus.

An exemplary sequence of the $DX_1EX_2X_3X_4H$ sequence is "DIETLYH". This sequence is perfectly preserved in thermostable DNA polymerases derived from Pyrococcus kodakaraensis KOD1 and Pyrococcus furiosus. Similarly, since the sequence in DNA polymerase derived from

Thermococcus litoralis is "DIETFYH", the sequence "DIETLYH" is completely preserved except that L is replaced by F (FIG. 1).

Further, it is easily anticipatable that variants wherein aspartic acid(D) and glutamic acid(E) in the $DX_1EX_2X_3X_4H$ sequence have been replaced by other amino acids are capable of producing similar effects as achieved by the present invention and such variants are included in the present invention.

The "other amino acids" are not particularly limited and include, for example, aspartic acid, glutamic acid and like acidic amino acids; tyrosine, alanine, glycine, valine, leucine, isoleucine, serine, proline, asparagine, glutamine, threonine, cysteine, methionine, tryptophan, phenylalanine and like neutral amino acids; lysine, arginine and like basic amino acids. Particularly preferred are aspartic acid, glutamic acid, tyrosine, alanine, lysine and arginine.

One embodiment of the present invention is a modified thermostable DNA polymerase having significantly reduced 3'-5' exonuclease activity as compared with the enzyme before modification, the reduction of 3'-5' exonuclease activity being achieved by the following modification: in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino

acid) of DNA polymerase having 3'-5' exonuclease activity, histidine(H) is replaced by an acidic amino acid such as glutamic acid or aspartic acid.

Another embodiment of the present invention is a modified thermostable DNA polymerase having improved amplifying efficiency, the improvement being achieved by the following modification: in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino acid) of DNA polymerase having 3'-5' exonuclease activity, histidine(H) is replaced by an acidic amino acid such as aspartic acid or glutamic acid or a neutral amino acid such as tyrosine or alanine.

A further embodiment of the present invention is a modified thermostable DNA polymerase having significantly improved 3'-5' exonuclease activity and/or fidelity on a DNA replication (amplification), the improvement being achieved by the following modification: in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino acid) of DNA polymerase having 3'-5' exonuclease activity, histidine(H) has been replaced by a basic amino acid such as lysine or arginine.

More specifically, according to the present invention, 3'-5' exonuclease activity can be reduced by replacing histidine(H) in the $DX_1EX_2X_3X_4H$ sequence (D:

aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino acid) of thermostable DNA polymerase having 3′-5′ exonuclease activity by an acidic amino acid such as aspartic acid or glutamic acid.

Of the variants of the KOD DNA polymerase, variant HE of KOD DNA polymerase (wherein histidine at the 147-position has been replaced by glutamic acid) and variant HD of KOD DNA polymerase (wherein histidine at the 147-position has been replaced by aspartic acid) actually showed about 25% and about 6.25% of 3′-5′ exonuclease activity of naturally occurring KOD DNA polymerase, respectively (FIG. 2).

According to the present invention, PCR amplification efficiency from low copy number of template DNA can especially be improved by replacing histidine(H) in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino acid) of thermostable DNA polymerase having 3′-5′ exonuclease activity by an acidic amino acid such as glutamic acid or aspartic acid or a neutral amino acid such as tyrosine or alanine.

Of the variants of the KOD DNA polymerase, variant HE (wherein histidine(H) at the 147-position has been replaced by glutamic acid(E)), variant HD (wherein histidine(H) at the 147-position has been replaced by

aspartic acid(D)), variant HY (wherein histidine(H) at the 147-position has been replaced by tyrosine(Y)) and variant HA (wherein histidine(H) at the 147-position has been replaced by alanine(A)) actually showed improved PCR efficiency (FIGs. 3 and 4). Of these variants, particularly variant HY did not show significant reduction in exonuclease activity (FIG. 2), which suggests that histidine at the 147-position affects PCR efficiency independently of its exonuclease activity level. In the amplification of long DNA fragments, especially improved PCR efficiency was observed in variants HE and HD wherein histidine had been replaced by an acidic amino acid (FIG. 4).

Further, according to the present invention, 3′-5′ exonuclease activity and/or PCR fidelity of thermostable DNA polymerase can be improved by replacing histidine(H) in the $DX_1EX_2X_3X_4H$ sequence of thermostable polymerase having 3′-5′ exonuclease activity by lysine, arginine and like basic amino acids.

Of the variants of KOD DNA polymerase, variant HK (wherein histidine(H) at the 147-position has been replaced by lysine(K)) and variant HR (wherein histidine(H) at the 147-position has been replaced by arginine(R)) according to the present invention actually showed remarkably increased 3′-5′ exonuclease activity

(FIG. 2). Both variants showed improved PCR fidelity as compared with the naturally occurring DNA polymerase (FIG. 5).

It is easily anticipatable that the improved function (enhanced amplication efficiency, improved fidelity on PCR, etc.) will similarly be produced by replacing histidine at the 147-position by an amino acid other than the above mentioned amino acids.

The DNA extension rate of the modified DNA polymerase of the invention is preferably at least 20 bases/second, more preferably at least 30 bases/second. More specifically, the DNA extension rate is about 20 to about 150 bases/second, preferably about 30 to about 150 bases/second.

The residual polymerase activity of the modified DNA polymerase of the invention after heat treatment (thermostability) is preferably 10% or more, preferably 40% or more, more preferably 60% or more. More specifically, the residual polymerase activity is about 10% to about 100%, preferably about 40% to about 100%, more preferably about 60% to about 100%.

A further embodiment of the present invention is a modified thermostable DNA polymerase wherein in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino acid) consisting

of DX_1E sequence within the EXO I region and a four amino acid length peptide adjacent to said glutamic acid(E) of thermostable DNA polymerase having 3′-5′ exonuclease activity, histidine(H) has been replaced by another amino acid,

the modified thermostable DNA polymerase having the following physicochemical properties:

(1) DNA extension rate: at least 20 bases/second; and

(2) thermostability: it is capable of maintaining 10% or more residual activity at pH 8.8 (determined at 25°C) after heat treatment at 95°C for 6 hours (that is, it is capable of retaining 10% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours).

Another embodiment of the present invention is a modified thermostable DNA polymerase wherein in the $DX_1EX_2X_3X_4H$ sequence (D: aspartic acid, E: glutamic acid, H: histidine, X_1, X_2, X_3 and X_4: any amino acid) consisting of DX_1E sequence within the EXO I region and four amino acid length peptide adjacent to said glutamic acid(E) of thermostable DNA polymerase having 3′-5′ exonuclease activity, histidine(H) has been replaced by another amino acid,

the modified thermostable DNA polymerase having the following physicochemical properties:

(1) DNA extension rate: at least 30 bases/second; and

(2) thermostability: it is capable of maintaining 40% or more residual activity at pH 8.8 (determined at 25°C) after heat treatment at 95°C for 6 hours (that is, it is capable of retaining 40% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours).

A further embodiment of the present invention is a modified thermostable DNA polymerase having the following physicochemical properties:

(1) DNA extension rate: at least 30 bases/second;

(2) thermostability: it is capable of maintaining 60% or more residual activity at pH 8.8 (determined at 25°C) after heat treatment at 95°C for 6 hours (that is, it is capable of retaining 60% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours);

(3) optimum temperature: about 65 to 75°C,

(4) molecular weight: about 89.97 kDa (calculated);

at any position other than the histidine site defined below in (5), one or more sugar chains may be deleted or added or one or more amino acids may be deleted, substituted, inserted or added.

(5) amino acid sequence: in the DIETLYH sequence (D: aspartic acid, I: isoleucine, E: glutamic acid, T:

threonine, L: leucine, Y: tyrosine, H: histidine) located at the 141- to 147-positions in the amino acid sequence of SEQ ID NO: 2, histidine(H) has been replaced by another amino acid.

A further different embodiment of the present invention is a modified thermostable DNA polymerase having the following physicochemical properties:

(1) DNA extension rate: at least 30 bases/second;

(2) thermostability: it is capable of maintaining 60% or more residual activity at pH 8.8 (determined at 25°C) after heat treatment at 95°C for 6 hours (that is, it is capable of retaining 60% or more DNA polymerase activity of untreated DNA polymerase at pH 8.8 (determined at 25°C) after treatment at 95°C for 6 hours);

(3) amino acid sequence: the amino acid at the 147-position in the amino acid sequence of SEQ ID NO: 2, namely, histidine(H) has been replaced by another amino acid.

A further embodiment of the invention is a modified thermostable DNA polymerase wherein histidine at the 147-position in the amino acid sequence of SEQ ID NO: 2 has been replaced by an amino acid selected from the group consisting of glutamic acid, aspartic acid, tyrosine, alanine, lysine and arginine.

In the present invention, "the amino acid

sequence of SEQ ID NO: 2" includes modified sequence of SEQ ID NO: 2 wherein one or more amino acids other than the histidine residue at the 147-position have been deleted, substituted or added, the modified sequence possessing DNA polymerase activity. Preferable examples of modified sequence of SEQ ID NO: 2 are ones showing 95% or more homology to the amino acid sequence of SEQ ID NO: 2 and possessing DNA polymerase activity, the modification being deletion, substitution or addition of one or more amino acids.

The present invention also provides a gene encoding the thermostable DNA polymerase as shown above.

Process for preparing modified DNA polymerase according to the invention

To produce these modified enzymes, any of the known methods can be used. For example, there is a technique which introduce a mutation in naturally occurring DNA polymerase so that a modified DNA polymerase having novel activity patterns is produced (*J. Biol. Chem.*, 264(11), 6447-6458(1989)).

The DNA polymerase-encoding gene in which a mutation is introduced is not particularly limited. Examples of genes include a gene derived from Pyrococcus kodakaraensis KOD1 and defined in SEQ ID NO: 3 in the

Sequence Listing; a gene derived from Pyrococcus furiosus (*Nucleic Acid Res.*, 21 (2), 259-265(1993)); and a gene derived from Thermococcus litoralis (*Proc. Natl. Acad. Sci. USA*, 89, 5577-5581(1992)). To mutate the naturally occurring DNA polymerase gene, any of the known methods can be used. For example, use can be made of a method comprising bringing a drug as a mutagen into contact with the naturally occurring DNA polymerase gene; UV radiation method; or protein engineering techniques such as PCR or site specific mutagenesis.

The QuickChange site-directed mutagenesis kit (Stratagene) used in the present invention makes use of the following steps:

(1) denaturing a plasmid having a target gene inserted therein and annealing mutagenesis primers to the target gene in said plasmid, followed by extending DNA using Pfu DNA polymerase,

(2) repeating the cycle described in (1) 15 times,

(3) selectively cleaving only the template plasmid by using, for example, restriction enzyme DpnI, which recognize the methylated base and

(4) transforming E. coli with a newly synthesized plasmid to provide a transformant containing the plasmid mutated as desired.

The modified DNA polymerase gene obtained as

described above may be subcloned into an expression vector, if necessary. For example, E. coli is transformed with the expression vector and plated on a agar medium containing a drug such as ampicillin to form a colony. The colony is inoculated onto a nutrient medium such as LB medium or 2 x YT medium and cultured at 37°C for 12 to 20 hours. The cultured bacteria were homogenized to extract a crude enzyme solution. Preferable vectors are pLED-MI, pBluescript, or their derivatives.

To homogenize the cultured bacteria, any of the known methods may be used and include, for example, ultrasonication, French Press (High Pressure Homogenizer) , glass bead disruption and like physical disruption, or lysis using a lytic enzyme such as lysozyme. The crude enzyme solution is thermally treated, e.g., at 80°C for 30 minutes to inactivate polymerases derived from the host. After adjusted the DNA polymerase activities of the mutants, 3'-5' exonuclease activities were measured and compared with that of naturally occurring DNA polymerase in order to estimate changing in their 3'-5' exonuclease activities.

To produce a purified DNA polymerase from the strain selected in this manner, any of the known means may be used and include, for example, the following method: The microorganism cultured in media is recovered and

treated enzymatically or by physical means so that a crude enzyme solution is extracted. The crude enzyme extract is subjected to heat treatment, e.g., at 80°C for 30 minutes and the DNA polymerase fraction is recovered by precipitation with sulfate ammonium. This crude enzyme fraction can be desalted by, e.g., gel filtration on Sephadex G-25 (Amersham Pharmacia Biotech).

After this procedure, the desalted enzyme is separated and purified by column chromatography such as Q-Sepharose or heparin-Sepharose to give a purified enzyme preparation. In this process, the enzyme preparation can be purified to such a degree that it shows an almost single band in SDS-PAGE.

By carrying out PCR amplification using the obtained enzyme, PCR efficiency can be evaluated from the occurrence or degree of amplification, and fidelity of DNA replication can also be evaluated.

The modified DNA polymerases of the present invention have excellent DNA amplification efficiency and high amplification fidelity and are suitable for use in PCR.

Nucleic acid amplification or extension method according to the invention

The nucleic acid amplification or extension

method of the present invention includes a method for synthesizing a DNA primer extension product by reacting DNA as template, one or more kinds of primers and dNTP (i.e., four types of deoxyribonucleoside triphosphates) using the modified thermostable DNA polymerase of the present invention to extend the primers.

There is no specific limitation on the primers in the present invention; however, they should be complementary or substantially complementary to the template DNA.

The method of the invention includes methods for extending nucleic acid using one primer. Such method includes primer extension methods and sequencing methods (including isothermal sequencing and cycle sequencing).

The method of the invention includes methods of amplifying nucleic acid by the PCR method using two or more kinds of primers. Preferably, the primers are 2 kinds of oligonucleotides and each of the primers is complementary to a DNA extension product of the other primer. It is preferable that heating and cooling be carried out repeatedly.

More specifically, the DNA amplification method using PCR is a method which comprises repeating a 3-step cycle comprising denaturation, annealing and extension in the presence of the template DNA, 4 types of

deoxyribonucleoside triphosphates, a pair of primers and the modified thermostable DNA polymerase of the present invention to exponentially amplify the template DNA region positioned between the pair of primers (*Nature*, 324 (6093), 13-19(1986)). Stated more specifically, a nucleic acid sample is denatured in the denaturation process; in the following annealing process, each primer is hybridized to a single-stranded template DNA region which is complementary to the primer; in the subsequent extension process, new DNA chains complementary to the single strand template DNA region are extended from each primer by the action of DNA polymerase to provide double-stranded DNA. One double-stranded DNA is amplified to give two double-stranded DNA fragments per cycle. Therefore, if this cycle is repeated n times, the sample DNA region between the pair of primers is theoretically amplified 2^n times.

To maintain the activities of the modified DNA polymerase of the invention, it is preferable that divalent ions such as magnesium ions and monovalent ions such as ammonium ions and/or potassium ions are present together with the polymerase of the invention. Further, the PCR reaction solution may include, in addition to such ions, BSA, a nonionic surfactant (e.g. Triton X-100) and a buffer solution. Useful buffer solutions include, for example, good buffers such as Tris and HEPES, and

phosphate buffers.

The PCR can be carried out, for example, by repeating a cycle using 3 different temperatures. More specifically, a solution containing a reaction buffer solution (120mM Tris-HCl (pH 8.0), 10mM KCl, 6mM $(NH_4)_2SO_4$, 0.1% TritonX-100, 10 µg/ml BSA), 0.4 pmol/µl each of primers, 0.2 mM dNTPs, 0.2 ng/µl template DNA and 0.05 u/µl modified DNA polymerase of the invention is reacted at 94°C for 15 seconds, 65°C for 2 seconds and 74°C for 30 seconds; and this cycle is repeated about 25 times.

<u>Reagent kit for amplifying nucleic acid according to the invention</u>

The reagent kit for amplifying nucleic acid of the present invention comprises 2 kinds of primers, each of the primers being complementary to a DNA extension product of the other primer; dNTP; the modified thermostable DNA polymerase of the invention; divalent ion(s); monovalent ion(s) and a buffer solution. More specifically, the reagent kit for amplifying nucleic acid of the invention comprises 2 kinds of primers, each of the primers being complementary to a DNA extension product of the other primer; dNTP; the above-mentioned modified thermostable DNA polymerase; magnesium ion; at least one ion selected from the group consisting of ammonium ion and

potassium ion; BSA; nonionic surfactant(s) and buffer solution(s), as exemplified above.

Another embodiment of the reagent kit for amplifying nucleic acid of the present invention comprises 2 kinds of primers, each of the primers being complementary to a DNA extension product of the other primer; dNTP; the modified thermostable DNA polymerase of the invention; divalent ion(s); monovalent ion(s); buffer solution(s); and optionally an antibody capable of suppressing polymerase activity and/or 3'-5' exonuclease activity of the thermostable DNA polymerase of the invention. Examples of antibodies include monoclonal antibodies, polyclonal antibodies and the like. The reagent kit for amplifying nucleic acid of the present invention is especially effective for enhancing PCR sensitivity and reducing nonspecific amplification.

The reagent kit for amplifying nucleic acid of the present invention further includes core kits which do not contain any primer contained in the above reagent kits.

<u>Reagent kit for producing a mutated DNA according to the invention</u>

The reagent kit for producing a mutated DNA of the present invention comprises mutagenesis primers, each of the primers being complementary to a DNA extension

product of the other primer; dNTP; and the modified thermostable DNA polymerase of the present invention. The reagent kit may further include divalent ion(s), monovalent ion(s) and buffer solution, as exemplified above.

According to the present invention, each mutagenesis primer consists of about 20 to about 150 bases and has a mutation (e.g., insertion, deletion or substitution), namely, a mutated site different from the template DNA sequence, near the midpoint of the sequence.

The reagent kit for producing a mutated DNA of the present invention further includes core kits which do not contain any primer contained in the above reagent kits.

Usable as buffer solutions in the nucleic acid amplification reagent kit and the reagent kit for producing a mutated DNA of the present invention are, for example, good buffers such as Tris and HEPES, and phosphate buffers. More specifically, 10 to 200 mM of various buffers (at pH 7.5 to 9.0; determined at 25°C) may be used.

The concentration of divalent ions such as magnesium ions and manganese ions is preferably 0.5 to 2 mM in the reaction stage. The concentration of monovalent ions such as ammonium ions and potassium ions is

preferably about 10 to about 100 mM in the reaction stage.

The concentration of the modified DNA polymerase of the invention is preferably about 0.01 to about 0.1 unit/μl in the reaction stage. The concentration of each of the primers is about 0.2 to about 2 pmol/μl.

Further, the modified thermostable DNA polymerase of the invention can be used as a 3'-5' exonuclease mainly by inactivation or decreasing of its polymerase activity using chemical or genetic engineering techniques.

DNA polymerase composition of the invention

Another embodiment of the invention is a DNA polymerase composition comprising one or more types of modified thermostable DNA polymerases of the present invention as described above. For example, by mixing one or more types of modified thermostable DNA polymerases of the present invention with a DNA polymerase having lower 3'-5' exonuclease activity, there can be provided a composition useful for amplification of long chain (e.g., base length: 4 to 20kb) nucleic acid (e.g., long PCR). Actually, as a method for amplifying long chain nucleic acid, there is a report on a PCR method using mixed thermostable DNA polymerases (i.e. Taq DNA polymerase (3'-

5′ exonuclease(-)) and Pfu or Ti DNA polymerase (3′-5′ exonuclease(+)) (Barns, W. M. (1994) *Proc. Natl. Acad. Sci. USA*, 91, 2216-2220). In the present invention, a combination may be, for example, a combination of modified DNA polymerase of the invention with Taq polymerase or with Tth polymerase; and a combination of DNA polymerase of the invention having lower 3′-5′ exonuclease activity with DNA polymerase of the invention having higher DNA polymerase.

Other components that may be incorporated into the composition of the invention include, for example, a buffer, divalent ion(s), monovalent ion(s), an antibody to the DNA polymerase, etc.

The present invention is described below in more detail with reference to the Examples.

Reference Example 1

Cloning of DNA Polymerase Gene Derived from Hyperthermophilic Archaeon Strain KOD1

Hyperthermophilic archaeon, Pyrococcus kodakaraensis KOD1 strain isolated in Kodakara Island, Kagoshima Prefecture, Japan, was cultured at 95°C and then recovered. Genomic DNA from Pyrococcus kodakaraensis KOD1 strain was prepared by the conventional manner. Two kinds

of primers, i.e., 5'-GGATTAGTATAGTGCCAATGGSSGGCGA-3' and 5'-GAGGGCAGAAGTTTATTCCGAGCTT-3' (SEQ ID NO: 26 and SEQ ID NO: 27; S represents a mixture of C and G) were synthesized based on the conserved region amino acid sequence of DNA polymerase (Pfu DNA polymerase) derived from Pyrococcus furiosus. PCR was conducted using the two kinds of primers and the genomic DNA as a template.

The DNA fragment thus amplified by PCR was sequenced. From the nucleotide sequence thus determined, its amino acid sequence was deduced. Then, the genomic DNA from the KOD1 strain was treated with a restriction enzyme, and the digest was subjected to Southern hybridization with the above amplified DNA fragment as a probe to determine the size of a fragment coding for the DNA polymerase (about 4 to about 7 Kbp). Further, the DNA fragment of this size was recovered from agarose gel and inserted into plasmid pBluescript (Stratagene). The mixture thus obtained was transformed into Escherichia coli JM109 to prepare a library. Colony hybridization with the same probe as in the Southern hybridization was conducted so that a clone strain (E. coli JM109/pBSKOD1) considered to contain the DNA polymerase gene derived from the KOD1 strain was obtained from the library.

Plasmid pBSKOD1 was recovered from the obtained clone strain and sequenced in the usual manner. Its amino

acid sequence was deduced from the nucleotide sequence thus determined. The DNA polymerase gene derived from the KOD1 strain consisted of 5010 bases and encoded 1670 amino acids (SEQ ID NO: 1).

2 intervening sequences (1374 to 2453 bp and 2709 to 4316 bp) were removed by PCR fusion method to prepare a complete gene fragment which is free of the intervening sequences, has an EcoRV site at the N-terminal and a BamHI site at the C-terminal, and encodes the DNA polymerase derived from the KOD1 strain (SEQ ID NO: 3). Further, this gene was subcloned in expression vector pET-8c capable of inducing expression of the gene under T7 promoter. A recombinant expression vector (pET-pol) was thus obtained. E. coli BL21 (DE3)/pET-pol has been deposited as FERM BP-5513 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

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Example 1

Subcloning of the KOD Polymerase Gene

To modify thermostable DNA polymerase, the KOD DNA polymerase gene was cut out of plasmid pET-pol and subcloned in plasmid pBluescript in the following manner. The KOD DNA polymerase gene, about 2.3 kb long, was cut out by digesting plasmid pET-pol with restriction enzymes

XbaI and BamHI (manufactured by Toyobo Co., Ltd.). A ligation kit (Ligation high, manufactured by Toyobo Co., Ltd.) was then used for ligation of this DNA fragment into plasmid pBluescript SK(-) previously digested with XbaI and BamHI. Then, the resulting plasmid was transformed into competent cells (competent high JM109, manufactured by Toyobo Co., Ltd.). The transformant was cultured at 35°C for 16 hours in an LB agar medium containing 100 µg/ml ampicillin (1% Bacto-trypton, 0.5% yeast extract, 0.5% sodium chloride, 1.5% agar; manufactured by Gibco), and a plasmid was prepared from the resulting colonies. From its partial nucleotide sequence, this plasmid was confirmed to carry the KOD DNA polymerase gene and designated plasmid pKOD1.

Example 2

Preparation of Modified Gene (HE) and Purification of Modified Thermostable DNA Polymerase (variant HE)

Plasmid pKOD1 obtained in Example 1 was used to prepare a plasmid (pKOD HE) carrying a gene encoding a modified thermostable DNA polymerase of KOD DNA polymerase in which histidine(H) at the 147-position had been replaced by glutamic acid(E). To prepare the plasmid, a QuickChange site-directed mutagenesis kit (Stratagene) was used in accordance with the instruction manual. The

mutagenesis primers used were primers as shown in SEQ ID NO: 4 and SEQ ID NO: 5. The mutant was confirmed by determining its nucleotide sequence. E. coli JM109 was transformed with the resulting plasmid to give E. coli JM109 (pKOD HE).

The obtained E. coli JM109(pKOD HE) was cultured in the following manner. 6 L of sterilized TB medium containing 100 µg/ml ampicillin (described in *Molecular cloning*, 2nd edition, p.A.2) was introduced into a 10-L jar fermenter. Inoculated into this medium was E. coli JM109 (pKOD HE) which had been cultured at 37°C for 16 hours in 50 ml LB medium (1% Bacto-trypton, 0.5% yeast extract, 0.5% sodium chloride manufactured by Gibco) containing 100 µg/ml ampicillin (using a 500-ml Sakaguchi flask). The microorganism was grown at 35°C for 12 hours under aeration. The microorganism was recovered from the culture broth by centrifugation, then suspended in 400 ml of a disruption buffer (10 mM Tris-HCl (pH 8.0), 80 mM KCl, 5 mM 2-mercaptoethanol, 1 mM EDTA) and homogenized by French Press (High Pressure Laboratory Homogenizer (Rannie)) to give a cell lysate. The cell lysate was heated at 85°C for 30 minutes and centrifuged to remove insoluble debris. The supernatant was treated with polyethylene imine for removal of nucleic acids, then precipitated by adding sulfate ammonium and subjected to

chromatography on heparin-Sepharose. Finally, the solution was dialized against a storage buffer (50 mM Tris-HCl (pH 8.0), 50 mM potassium chloride, 1 mM dithiothreitol, 0.1% Tween 20, 0.1% Nonidet P40, 50% glycerin) so that the modified thermostable DNA polymerase (variant HE) was obtained. In the purification described above, the measurement of DNA polymerase activity was conducted in the manner as shown below. When the enzyme activity was high, the sample was measured after dilution.

(Reagent)

A: 40 mM Tris-HCl (pH 7.5)
16 mM magnesium chloride
15 mM dithiothreitol
100 µg/ml BSA

B: 2 µg/µl activated calf thymus DNA

C: 1.5 mM dNTP (250 cpm/pmol [^{3}H] dTTP)

D: 20% trichloroacetic acid
(2 mM sodium pyrophosphate)

E: 1 mg/ml salmon sperm DNA

(Method)

25 µl of Solution A, 5 µl each of Solutions B and C, and 10 µl sterilized water were added to a microtube and mixed by stirring. Then, 5 µl of the purified enzyme

solution (optionally diluted) was added to the mixture and reacted at 75°C for 10 minutes. The reaction mixture was cooled and 50 µl of Solution E and 100 µl of Solution D were added and stirred, followed by further cooling with ice for 10 minutes. This solution was filtered through a glass filter (Wattman GF/C filter), followed by extensive washing with Solution D and ethanol, and radioactivity of the filter is counted in a liquid scintillation counter (manufactured by Packard) to determine the incorporation of the nucleotide into the template DNA. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the incorporation of 10 nmole of nucleotides into acid-insoluble fraction per 30 minutes under the above-mentioned conditions.

Example 3

Preparation of Modified Gene (HD) and Purification of Modified Thermostable DNA Polymerase (variant HD)

A plasmid (pKOD HD) carrying a gene encoding a modified thermostable DNA polymerase of the KOD DNA polymerase in which histidine(H) at the 147-position had been replaced by aspartic acid(D) was prepared in the same manner as in Example 2. The mutagenesis primers used were primers as shown in SEQ ID NO: 6 and SEQ ID NO: 7. Further, the modified thermostable DNA polymerase (variant

HD) was obtained using the same purification method as in Example 2.

Example 4

Preparation of Modified Gene (HY) and Purification of Modified Thermostable DNA Polymerase (variant HY)

A plasmid (pKOD HY) carrying a gene encoding a modified thermostable DNA polymerase of the KOD DNA polymerase in which histidine(H) at the 147-position had been replaced by tyrosine(Y) was prepared in the same manner as in Example 2. The mutagenesis primers used were primers as shown in SEQ ID NO: 8 and SEQ ID NO: 9. Further, the modified thermostable DNA polymerase (variant HY) was obtained using the same purification method as in Example 2.

Example 5

Preparation of Modified Gene (HA) and Purification of Modified Thermostable DNA Polymerase (variant HA)

A plasmid (pKOD HA) carrying a gene encoding a modified thermostable DNA polymerase of the KOD DNA polymerase in which histidine(H) at the 147-position had been replaced by alanine(A) was prepared in the same manner as in Example 2. The mutagenesis primers used were primers as shown in SEQ ID NO: 10 and SEQ ID NO: 11.

Further, the modified thermostable DNA polymerase (variant HA) was obtained using the same purification method as in Example 2.

Example 6

Preparation of Modified Gene (HK) and Purification of Modified Thermostable DNA Polymerase (variant HK)

A plasmid (pKOD HK) carrying a gene encoding a modified thermostable DNA polymerase of the KOD DNA polymerase in which histidine(H) at the 147-position had been replaced by lysine(K) was prepared in the same manner as in Example 2. The mutagenesis primers used were primers as shown in SEQ ID NO: 12 and SEQ ID NO: 13. Further, the modified thermostable DNA polymerase (variant HK) was obtained using the same purification method as in Example 2.

Example 7

Preparation of Modified Gene (HR) and Purification of Modified Thermostable DNA Polymerase (variant HR)

A plasmid (pKOD HR) carrying a gene encoding a modified thermostable DNA polymerase of the KOD DNA polymerase in which histidine(H) at the 147-position had been replaced by arginine(R) was prepared in the same manner as in Example 2. The mutagenesis primers used were

primers as shown in SEQ ID NO: 14 and SEQ ID NO: 15. Further, the modified thermostable DNA polymerase (variant HR) was obtained using the same purification method as in Example 2.

Example 8

Preparation of Modified Gene (HS) and Purification of Modified Thermostable DNA Polymerase (variant HS)

A plasmid (pKOD HS) carrying a gene encoding a modified thermostable DNA polymerase of the KOD DNA polymerase in which histidine(H) at the 147-position had been replaced by serine(S) was prepared in the same manner as in Example 2. The mutagenesis primers used were primers as shown in SEQ ID NO: 16 and SEQ ID NO: 17. Further, the modified thermostable DNA polymerase (variant HS) was obtained using the same purification method as in Example 2.

Example 9

Preparation of Modified Gene (HQ) and Purification of Modified Thermostable DNA Polymerase (variant HQ)

A plasmid (pKOD HQ) carrying a gene encoding a modified thermostable DNA polymerase of the KOD DNA polymerase in which histidine(H) at the 147-position had been replaced by glutamine(Q) was prepared in the same

manner as in Example 2. The mutagenesis primers used were primers as shown in SEQ ID NO: 18 and SEQ ID NO: 19. Further, the modified thermostable DNA polymerase (variant HQ) was obtained using the same purification method as in Example 2.

Example 10

Comparison of 3'-5' Exonuclease Activity between Modified Thermostable DNA Polymerases

The exonuclease activities of the modified thermostable DNA polymerases obtained in Examples 2 to 9 and variants IK and IQ were determined in the following manner. The variants IK and IQ were prepared from KOD DNA polymerase described in Japanese Unexamined Patent Publication No. 1998-42871 by replacing isoleucine at the 142-position with lysine and glutamine respectively in accordance with the method described in Japanese Unexamined Patent Publication No. 1998-42871 (namely, variants IK and IQ were the KOD DNA polymerase-variants in which isoleucine(I) at the 142-position have been replaced by lysine(K) and glutamine(Q),respectively.) As a control, the naturally occurring KOD DNA polymerase (Toyobo Co., Ltd.) was used.

50 µl of a reaction solution (120 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 6 mM ammonium sulfate, 1 mM

$MgCl_2$, 0.1% Triton X-100, 0.001% BSA, 5 µg tritium-labeled E. coli DNA) was pipetted into each of 1.5-ml microtubes. 0.06U or 0.025U of each DNA polymerase was added to the reaction mixture. The mixture was reacted at 75°C for 10 minutes and then cooled with ice to terminate the reaction. After 50 µl of 0.1% BSA was added as a carrier, 100 µl of a solution containing 10% trichloroacetic acid and 2% sodium pyrophosphate was further added and mixed. The mixture was left on ice for 15 minutes and then centrifuged at 12,000 r.p.m. for 10 minutes to separate precipitates. The radioactivity of 100 µl of the supernatant was measured in a liquid scintillation counter (Packard) whereby the amount of the nucleotide delivered into the acid-soluble fraction was determined. Based on the radioactivities from 0.06U and 0.025U (polymerase unit) of mutated enzymes, relative 3'-5' exonuclease activities were estimated. FIG. 2 shows relative exonuclease activities of the DNA polymerases.

The results proved that thermostable DNA polymerases with 3'-5' exonuclease activity at different levels can be produced by the present invention. As compared with the naturally occurring KOD DNA polymerase (100%), the modified thermostable DNA polymerases had 3'-5' exonuclease activity at the following levels: variant HD had about 6.25%; variant HE about 25%; variant HY about

90%; variant HA about 30%; variant HS about 50%; variant HQ about 50%; variant HK about 400%; variant HR about 300%; variant IK about 6.25%; and variant IQ about 25%.

Example 11 Confirmation of Thermostability

The thermostability of the modified thermostable DNA polymerases obtained in Examples 2, 3 and 6 was determined in the following manner. 5 units of each purified modified DNA polymerase was mixed with 100 µl of a buffer solution (20 mM Tris-HCl pH 8.8 at 25°C, 10 mM potassium chloride, 10 mM ammonium sulfate, 2 mM magnesium sulfate, 0.1% Triton X-100, 0.1 mg/ml BSA, 5 mM 2-mercaptoethanol) and pre-incubated at 95°C. A sample was recovered from this mixture with time, and its polymerase activity was determined in the method described in Example 2. For comparison, a naturally occurring KOD DNA polymerase (Toyobo Co., Ltd.) and Taq DNA polymerase were also subjected to the same procedure. Table 1 shows that similar to the naturally occurring KOD DNA polymerase, any of the modified thermostable DNA polymerases had 60% or more residual activity after treatment at 95°C for 6 hours.

Table 1

DNA polymerase	Residual DNA polymerase activity
Naturally occurring KOD DNA polymerase	70%
Example 2	84%
Example 3	77%
Example 6	68%
Taq DNA polymerase	10%

Example 12 Measurement of DNA Extension Rate

The modified thermostable DNA polymerases obtained in Examples 2, 3, 6 and 7 were examined for DNA extension rate in the following manner. 1 unit of each purified modified DNA polymerase was reacted with single stranded M13mp18 DNA to which 0.2 µg of the primer (SEQ ID NO: 28) had been annealed. The reaction was carried out in 10 µl of a reaction solution (20 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride, 7.5 mM dithiothreitol, 100 µg/ml BSA, 0.1 mM dNTP, 0.2 µCi [α-^{32}P]dCTP) at 75°C for 20, 40, and 60 seconds respectively. The reaction was terminated by adding an equal volume (equal to the reaction mixture) of a reaction-terminating solution (50 mM sodium hydroxide, 10 mM EDTA, 5% Ficoll, 0.05% Bromophenol Blue). For comparison, Pfu DNA polymerase (Stratagene) and the naturally occurring KOD DNA polymerase (Toyobo Co., Ltd.)

were subjected to the same procedure.

The DNA fragments are sized by electrophoresis on an alkaline agarose gel, and the gel was dried and subjected to autoradiography. As a DNA size marker, labeled λ/HindIII was used. The DNA extension rate was determined by measuring the size of the synthesized DNA using a band of this marker as an indicator. Table 2 shows the results. Similar to the naturally occurring KOD DNA polymerase, any of the modified DNA polymerases had an extension rate of about 120 bases/second. By contrast, Pfu DNA polymerase had an extension rate of about 20 bases/second.

Table 2

DNA polymerase	Extension rate (base/sec.)
Naturally occurring KOD DNA polymerase	120
Example 2	123
Example 3	123
Example 6	123
Example 7	120
Pfu DNA polymerase	20

Example 13 PCR (1) by Use of Modified DNA Polymerases

PCR was carried out using naturally occurring

KOD DNA polymerase (hereinafter sometimes referred to as "WT") and modified thermostable DNA polymerases (variants HE, HD, HY, HA, HK, HR, IK and IQ). The variants IK and IQ were prepared from KOD DNA polymerase described in Japanese Unexamined Patent Publication No. 1998-42871 by replacing isoleucine(I) at the 142-position with lysine(K) and glutamine(Q) respectively. It is known that variants HE and IQ have a similar level of 3′-5′exonuclease activity and variants HD and IK have a similar level of 3′-5′exonuclease activity.

1 µl (1U/µl) of each enzyme was added to 49 µl of a reaction solution (1 x KOD-Plus-buffer (Toyobo Co., Ltd.), 1 mM $MgSO_4$, 0.2 mM dNTP, 100 ng and 10 ng K562 DNA (Life Technologies, Inc.), and 10 pmol each of primers shown in SEQ ID NO: 20 and SEQ ID NO: 21). Using PCR system GeneAmp2400 (Perkin-Elmer Corp.) as a thermal cycler, the PCR amplification reaction was conducted under the following conditions. The reaction was carried out at 94°C for 2 minutes, followed by a cycle consisting of reaction at 94°C for 15 seconds, at 60°C for 30 seconds and at 68°C for 3 minutes and 30 seconds. This cycle was repeated 30 times. After completion of the reaction, 10 µl of the reaction solution was subjected to agarose gel electrophoresis and dyed with ethidium bromide. Under UV irradiation, amplification of about 3.6 kb target DNA

fragment was confirmed. FIG. 3 shows the result of agarose gel electrophoresis. The result proved that as compared with the naturally occurring KOD DNA polymerase, variants HE, HD, HY and HA especially improve PCR amplification of low copy number of template DNA (10 ng).

Example 14 PCR (2) by Use of Modified DNA Polymerase

Using the modified thermostable polymerases, variants HE, HD, HY and HA which had achieved good results in Example 13, amplification of larger size DNA was attempted.

1 µl (1U/µl) of each enzyme was added to 49 µl of a reaction solution (1 x KOD-Plus-buffer (Toyobo Co., Ltd.), 1 mM $MgSO_4$, 0.2 mM dNTP, 100 ng and 50 ng K562 DNA (Life Technologies, Inc.), and 10 pmol each of primers shown in SEQ ID NO: 22 and SEQ ID NO: 23). Using PCR system GeneAmp2400 (Perkin-Elmer Corp.) as a thermal cycler, the PCR amplification reaction was conducted under the following conditions. The reaction was carried out at 94°C for 2 minutes, followed by a cycle consisting of reaction at 94°C for 15 seconds, at 60°C for 30 seconds and at 68°C for 6 minutes. This cycle was repeated 30 times. After completion of the reaction, 10 µl of the reaction solution was subjected to agarose gel electrophoresis and dyed with ethidium bromide. Under UV

irradiation, amplification of about 6.2 kb target DNA fragment was confirmed. Variants HD and HE produced especially good amplification results (FIG. 4). Amplification using naturally occurring KOD DNA polymerase (WT) could not be detected (not shown in FIG. 4).

Example 15

Measurement of modified KOD DNA polymerase fidelity

The fidelity of naturally occurring KOD DNA polymerase and modified thermostable DNA polymerases was measured in the following manner. 1 µl (1U/µl) of each enzyme was added to 49 µl of a reaction solution (1 x KOD-Plus-buffer (Toyobo Co., Ltd.), 1 mM $MgSO_4$, 0.2 mM dNTP, 2.5 ng plasmid pMol 21 (*Journal of Molecular Biology* (1999) 289, 835-850), and 10 pmol each of primers shown in SEQ ID NO: 24 and SEQ ID NO: 25). The PCR amplification reaction was conducted under the following conditions using variants HD, HE, HY, HA, HK and HR among the obtained variants, and variants IK and IQ described in Japanese Unexamined Patent Publication No. 1998-42871. Using PCR system GeneAmp2400 (Perkin-Elmer Corp.) as a thermal cycler, the PCR was carried out under the following conditions. The reaction was carried out at 94°C for 2 minutes, followed by a cycle consisting of reaction at 94°C for 15 seconds, at 60°C for 30 seconds

and at 68°C for 4 minutes. This cycle was repeated 25 times. At the same time, an amplification reaction using rTaq DNA polymerase was carried out under the following reaction conditions. 0.5 µl (5 U/µl) of Taq DNA polymerase was added to 49 µl of a reaction solution (1 x rTaq buffer (Toyobo Co., Ltd.), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 ng plasmid pMol 21 (*Journal of Molecular Biology* (1999) 289, 835-850), and 10 pmol each of primers shown in SEQ ID NOS: 24 and 25). The PCR reaction was carried out at 94°C for 2 minutes, followed by a cycle consisting of reaction at 94°C for 15 seconds, at 60°C for 30 seconds and at 68°C for 5 minutes. This cycle was repeated 25 times.

After completion of PCR, the reaction mixture was treated with phenol/chloroform and DNA was precipitated with ethanol. The precipitates were dissolved in 100 µl of distilled water. To the solution were added 10 µl of avidin magnetic beads (manufactured by DYNAL). The mixture was subjected to invert blending for 30 minutes. The magnetic beads were concentrated using a magnetic separation stand (Magical Trapper; product of Toyobo Co., Ltd.). After discarding the supernatant, 100 µl of wash A (10mM Tris-HCl, (pH 8.0), 1mM EDTA and 1M NaCl) was added to the magnetic beads, followed by stirring for 10 seconds and the magnetic beads were

concentrated again using the magnetic separation stand and the supernatant was discarded (washing process). The washing process was repeated once again and then the magnetic beads were washed with wash B(10mM Tris-HCl (pH 8.0), 1mM EDTA). Only the magnetic beads were collected using the magnetic separation stand and subsequently, 40 µl of distilled water, 5 µl of a restriction enzyme buffer solution and 50 U of restriction enzyme Mlu I (Toyobo Co., Ltd.) were added to the beads. The treatment was allowed to proceed with invert-mixing at 37°C for 3 hours. Then, the magnetic beads were concentrated again using the magnetic separation stand and only the supernatant was collected. The collected DNA solution was desalted by the ethanol precipitation method. To a 10 ng quantity of the desalted solution was added a ligation reagent (Ligation high, manufactured by Toyobo Co., Ltd.). A ligation reaction was allowed to proceed at 16°C for 16 hours. The resulting DNA was transformed into competent cells of E. coli MF-101 (*Journal of Molecular Biology* (1999) 289, 835-850) prepared by the method described in *Molecular cloning* 2nd edition 1.74-1.81.

The transformed E. coli solution was divided into two. One was cultured on an LB agar medium (0.6%) <plate A> containing 200 µg/ml of ampicillin at 30°C for 24 hours, whereas the other was cultured on an LB agar

medium (0.6%) <plate B> containing 200 μg/ml of ampicillin and 400 μg/ml of streptomycin at 30°C for 24 hours. The number of colonies appearing on the plates were counted. Mutation frequency (%) was calculated by dividing the number of colonies on plate B by the number on plate A and expressed in percentage terms (multiplied by 100). A lower mutation frequency indicates a higher DNA polymerase fidelity in DNA replication.

Fig. 5 shows the results. rTaq polrmerase free of 3′-5′ exonuclease (proof-reading) activity showed a high mutation frequency of 7.91%. By contrast, all the variants obtained according to the present invention and naturally occurring DNA polymerase (WT) showed 1% or less mutation frequency. Among them, variants HK and HR having increased 3′-5′ exonuclease activity as compared with naturally occurring DNA polymerase showed mutation frequency of 0.12% and 0.17% respectively, which are remarkable good values as compared with a mutation frequency of 0.47% achieved with naturally occurring DNA polymerase.

As shown above, the present invention achieved production of thermostable DNA polymerases with different levels of DNA amplification efficiency, 3′-5′ exonuclease activity and fidelity. The method of the present

invention comprising modification of conventional archaebacteria-derived thermostable DNA polymerases produces modified thermostable DNA polymerases that are useful for various purposes such as long template amplification and high fidelity amplification.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the EXO I region (underlined) and amino acid sequence adjacent to the EXO I region in various DNA polymerases.

FIG. 2 shows relative 3'-5' exonuclease activities in various KOD DNA polymerase variants (calculated relative to the activity of WT as 100).

FIG. 3 shows the result of PCR amplification of β-globin gene (3.6kb) using human genome DNA as a template and various KOD DNA polymerase variants.

A: PCR using 100 ng of human cell line K562-derived DNA

B: PCR using 10 ng of human cell line K562-derived DNA

1: naturally occurring DNA polymerase (WT),

2: variant HD,

3: variant HE,

4: variant HY,

5: variant HA,

6: variant HK,

7: variant HR,

8: variant IK,

9: variant IQ.

FIG. 4 shows the result of the PCR amplification of Myosin heavy chain gene (6.2kb) using human genome DNA as a template and various modified KOD DNA polymerases.

PCR using 50 ng of human cell line K562-extracted DNA

1: variant HD,

2: variant HE,

3: variant HY,

4: variant HA.

Fig. 5 shows mutation frequency (%) in PCR amplification using various KOD DNA polymerase variants.